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BEFORE THE BOARD OF APPEALS AND INTERFERENCES
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Serial No. 10/613,380 | Customer No. 23379

Applicant: Wendell Lim, John Dueber, Brian Yeh | Confirmation No. 5261

Filed: Jul 03, 2003 | Group Art Unit: 1631

Docket No. UCSF03-114 | Examiner: Skibinsky, Anna

Title: *Protein Logic Gates*

CERTIFICATE OF TRANSMISSION
I hereby certify that this corr is being transmitted by facsimile to the Comm for Patents 571-273-8300 on December 3, 2006.
Signed
Richard Aron Osman

BRIEF ON APPEAL

The Honorable Board of Appeals and Interferences
United States Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Honorable Board:

We appeal from the Nov 30, 2006 Examiner's final rejection of claims 1, 2, 6 and 8.

REAL PARTY IN INTEREST

The real party in interest is the Regents of the University of California, the assignee of this application.

RELATED APPEALS AND INTERFERENCES

Appellants are unaware of any related appeals or interferences.

STATUS OF CLAIMS

Claims 1-11 and 14-22 are pending in the application. Claims 3-5, 7, 9-11 and 15-22 are withdrawn from consideration; claim 14 is objected to; and claims 1, 2, 6 and 8 are rejected and subject to this appeal.

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PAGE 1/18 * RCVD AT 12/3/2006 9:41:39 PM [Eastern Standard Time] * SVR:USPTO-EFXRF-3/12 * DNIS:2738300 * CSID:949 218 1767 * DURATION (mm-ss):08-32

STATUS OF AMENDMENTS

All Amendments are believed to be properly before the Board.

SUMMARY OF CLAIMED SUBJECT MATTER

An autoregulated fusion protein comprising an output domain and a plurality of input domains, wherein at least one of the input domains is heterologous to the output domain, and the input domains interact with each other to allosterically and external ligand-dependently regulate the output domain. (Specification p. 5 lines 26-30; claim 1).

In particular embodiments, the output domain is catalytic (Specification p. 2, lines 8-11; claim 2); the plurality comprises four input domains, all heterologous to the output domain, and which form first and second specific binding pairs which allosterically regulate the output domain dependent on first and second, different external ligands, respectively (Specification p. 5, lines 4-8; claim 6); and the input domains cooperatively regulate the output domain as an AND-gate (Specification p. 5, lines 8-9; claim 8). In a specific embodiment the output domain is a Neuronal Wiskott-Aldrich Syndrome Protein (N-WASP) WA domain, and the input domains are (i) a PDZ domain and (ii) a SH3 domain (Specification p. 21, line 28 – p. 24, line 4; claim 14).

GROUND OF REJECTION TO BE REVIEWED ON APPEAL

I. WHETHER THE EXAMINER HAS PROPERLY REJECTED CLAIMS 1, 2, 6, and 8 UNDER 35USC112, FIRST PARAGRAPH (ENABLEMENT).

ARGUMENT

I. THE EXAMINER HAS NOT PROPERLY REJECTED CLAIMS 1, 2, 6, and 8 UNDER 35USC112, FIRST PARAGRAPH (ENABLEMENT).

The test for enablement is whether the specification enables one skilled in the art to practice the invention as claimed without undue experimentation. Here, the claimed invention is an autoregulated fusion protein comprising an output domain and a plurality of input domains, wherein at least one of the input domains is heterologous to the output domain, and the input domains interact with each other to allosterically and external ligand-dependently regulate the output domain. The fusion proteins link protein input and output domains that are normally not related to provide protein signaling switches analogous to logic gates with diverse and novel input/output properties.

Accordingly, the selected input and output domains are discretionary to the user according to intended use, and essentially any output domain providing a desired activity or binding affinity may be employed, so long as output activity can be regulated by ligand-dependent interaction of the input domains (e.g. Specification, p. 6, lines 13-15). Similarly the selection of input domains is user discretionary, so long as the selected domains interact to provide the requisite ligand-dependent gating of the output domain (e.g. Specification, p.7, lines 18-19).

For example, output domain functional compatibility with the fusion proteins is readily confirmed in routine activity screens (e.g. Specification, p.6, lines 16-17). A wide variety of output activities may be obtained, depending on the ultimate user application, including catalytic, label-generative, metabolic, apoptotic, and specific-binding output activities (Specification, p.6, lines 17-19). Table 1 lists exemplary output domains shown to have regulatable output activities, including well-studied kinase, phosphatase and protease domains (Specification, p.6, line 25 – p.7, line 7).

Similarly, input domain functional compatibility (demonstrating gating behavior) with the fusion proteins is readily confirmed in routine activity screens. A wide variety of interacting input domains may be used, depending on the ultimate user application, including peptide hormones and cognate receptor ligand binding domains (LBD), immune receptors and cognate antigenic peptides, src-homology domains and cognate peptide ligands, and various catalytic input domains, including modular proteases and both cleavable and non-cleavable pseudosubstrate peptides, modular kinases and peptide substrates, modular phosphotases and phospho-peptide substrates, etc. The input domain interaction can be provided by homo- or hetero-dimerization, by specific pair binding, by higher order complex formation, by enzyme-substrate catalysis (e.g. phosphorylation, glycosylation, prenylation, acylation, lipid modification, etc.). Specification, p.7, lines 18-30.

Preferred input domains comprise native, modular interacting domains which mediate binding of naturally interacting proteins, or natural, modular receptors or enzymes and their cognate ligands and substrates. A wide variety of such modular interacting components has been identified, categorized and subject to grafting. In addition, suitable input domains may be derived from vast public databases of known interacting proteins, including Database of Interacting Proteins (DIP), Database of Ligand-Receptor Proteins, Java-based DIP, and LiveDIP; see, e.g. Xenarios, et al. (2002) NAR 30:303-5; Xenarios, et al.(2001) NAR 29:239-41; Xenarios

et al., (2000) NAR 28:289-91; Deane et al.(2002) Mol Cell Prot 1:349-356; Graeber et al. (2001) Nat.Genet. 29:295-300; Marcotte et al. (2001) Bioinformatics 17:359-63; Salwinski et al. (2003) Mol Cell Proteomics. 2002 May;1(5):349-56; Xenarios et al. (2001) Curr Opin Biotechnol 12:334-339. In addition, many protein interaction domains can be mutated to provide alternative specificity binding partners. For example, mutation of a threonine residue of the Src SH2 domain to tryptophan converts ligand-binding specificity from the Src-like pTyr-Glu-Glu-Ile (SEQ ID NO:1), to the signature Grb2 binding motif pTyr-X-Asn (Kimber et al. Molecular Cell 2000. 5, 1043-1049). Table 2 lists exemplary input domain binding pairs shown to have external ligand regulatable binding. Specification, p. 8, line 5 – p.18, line 22).

To promote their interactions, one or more of the input domains may be coupled to the fusion protein through a linker or spacer peptide. Linker peptides are widely used in fusion proteins. Linker sequence and length are user-discretionary, though the linkers should not interfere with the ouput domain when the switch is in the active state (e.g. de-repressed), which is readily confirmed empirically. Preferred linkers often provide structural flexibility and mobility to the input domain. Exemplary use of linker peptides is provided in the disclosed examples of exemplary fusion proteins. Specification, p.7, lines 31 – p.8, line 4.

The claimed protein switches are readily designed or screened such that external ligand activation up-regulates, down-regulates, or otherwise alters output activity. For example, activation can increase, decrease or alter label expression, binding or substrate affinity or specificity, etc. In particular embodiments, the output domain is constitutively active or functional, and in the absence of the ligand, the input domains interact to inhibit the output domain. Where the selected output domain also comprises a suitable input or interaction domain, this endogenous interaction domain may be exploited to create novel allostery in conjunction with a heterologous input or interaction domain. Typically, such endogenous input domains are positioned on the output domain so as to not interfere with the output activity, e.g. the output activity when the fusion protein is de-repressed with ligand. Specification, p.7, lines 8-17.

A wide variety of external ligands may be used to activate the switches by interacting with one or more of the input domains. The external ligands may activate reversibly, such as by reversible competitive or allosteric interaction with one or more of the input domains, or may activate irreversibly, such as through covalent modification. For example, in the case of an SH3 input domain, proline rich peptides can be used as both a second, integral input domain, and as

an external competitive ligand. Alternatively, the external ligand can comprise a kinase activity which phosphorylates (covalently modifying) the SH3 domain proximate to the proline-rich binding site, and thereby disrupts interaction of the input domains. Specification, p.18, line 24 – p.19, line 2.

In particular embodiments, the fusion proteins comprise two input domains, both heterologous to the output domain, and which form a specific binding pair. In these embodiments, the input domains may also be referred to as receptor-ligand pairs, wherein this internal ligand is one of the input domains, as opposed to the actuating, external ligand which competitively or allosterically disrupts pair-specific binding of the input domains. This input domain binding pair motif may be expanded with additional input domains to provide any desired form of cooperative or antagonistic regulation. For example, the fusion protein may comprise two or more specific binding pairs of input domains which provide higher-order cooperative gating behavior. Accordingly, depending on design or selection, multiple input domains can cooperatively regulate the fusion protein in a wide variety of functionalities, including as an OR-gate, an AND-gate, and an AND-NOT-gate. Similarly, a plurality of output domains can be combined in a single fusion protein, to provide more complex switching. Table 3 provides the compositions of exemplary fusion protein switches, including their corresponding output domain, input domains and regulating external ligand. Specification, p.19, lines 3-24.

The Specification plainly enables one skilled in the art to make and use without undue experimentation an autoregulated fusion protein comprising an output domain and a plurality of input domains, wherein at least one of the input domains is heterologous to the output domain, and the input domains interact with each other to allosterically and external ligand-dependently regulate the output domain. The Specification plainly enables one skilled in the art to make and use such fusion proteins with a wide range of alternative output and input domains. Swapping alternative input and output domains in the recited fusion proteins involves only routine gene splicing and activity screening. A finding of undue experimentation requires much, much more: "the mere fact that the experimentation may have been difficult and time consuming does not mandate a conclusion that such experimentation would have been considered to be 'undue' in this art. Indeed, great expenditures of time and effort were ordinary in the field of vaccine preparation." *Falkner et al. v. Inglis et al.* (448 F.3d 1357, Fed. Cir. 2006)), favorably quoting underlying Board Decision). Further, that one skilled in the art may engage in trial and error experimentation to practice a claimed invention does not necessarily amount to undue

experimentation (see e.g. Koito Manufacturing Co. v. Turn-Key-Tech, L.L.C., 381 F.3d 1142 (Fed. Cir. 2004)[1]. Hence, we can not acquiesce in the Action's proposal to eviscerate our invention by restricting the claims to but a single exemplified fusion protein having an N-WASP output domain and SH3 and PDZ input domains" (Action p.3, lines 1-3 of bottom para.).

Those skilled in the art recognize that Applicants teachings enable or "pave the way" for creating alternative signal-response elements by protein design:

> In an intriguing variation on this theme, Mark Ptashne nominated two articles from the same group, in which proteins were engineered to mediate novel cellular responses to a particular input. In one article from this group, by Park et al. (3), a yeast scaffolding protein was engineered to bind a novel combination of kinases, so that the pheromone α-factor, instead of inducing a mating response, initiated a response normally produced by exposure of cells to high osmolarity. In the second article, by Dueber et al. (4), variants of the actin-regulatory protein N-WASP (neuronal Wiskott-Aldrich signaling protein) were engineered so that they could be activated by a synthetic switch designed by the authors. In nominating this pair of papers, Ptashne noted, "These two remarkable papers show us how in two quite disparate cases, seemingly intricate and precisely defined protein-protein interactions can be replaced by simpler heterologous interactions without loss of function. These findings shed light on how these systems might have evolved and pave the way for creating new signal-response elements by protein design."

Adler et al. Signaling Breakthroughs of the Year. Adler, Gough, and Ray (2004) Science's STKE 2004: et1-1 (attached).

Applicants submit that the Specification enables one skilled in to practice the recited method without undue experimentation.

We have of record uncontroverted evidence in the form of an expert Declaration by Dr. Henry Bourne, averring to the foregoing, and confirming that the Specification provides adequate guidance to permit one skilled in the art to practice the invention as claimed without undue experimentation. Dr Bourne explains:

> In fact, following the guidance of their disclosure, the inventors have used modular intramolecular interaction domains to engineer novel regulatory control over distinct catalytic output activities. For example, they have demonstrated the ability to apply their

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[1] "Because Koito produced no evidence that the trial and error required to practice the claimed invention would be unduly laborious or beyond the reach of one of ordinary skill in the art, we affirm the district court's grant of Turn-Key's JMOL on the issue of enablement." (Koito Manufacturing Co. v. Turn-Key-Tech, 381 F.3d 1142, XXXX (Fed. Cir. 2004).

method of regulation to Dbl-homology (DH) domains of distinct guanine nucleotide exchange factors (GEFs). They have inserted the engineered GEF switches into cells and used them to precisely alter cell behavior, conferring a new morphological response when stimulated by an appropriated upstream target. They have also demonstrated that they can build these types of autoinhibitory switches that respond to diverse input stimuli. For example, they have engineered switch proteins that are activated by a specific protein kinase by using an autoinhibitory control unit that consists of a PDZ domain-peptide recognition pair whose interaction is disrupted by phosphorylation. They have also built switches with more complex behavior by combining multiple domains. For example, they have built switches that are controlled by 3 inputs simultaneously (by using 3 autoregulatory domain interactions) or switches that show digital type (all-or-none) responses by using multiple autoregulatory interactions of the same type. I am familiar with these types of protein engineering, and in my opinion all these results were predictably obtained following the guidance of their patent application without requiring any undue experimentation.

Appellants respectfully request reversal of the subject enablement rejection by the Board of Appeals. The appeal brief fee is provided in the accompanying PTO-2038.

Respectfully submitted,
Science & Technology Law Group

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CLAIMS APPENDIX

1. An autoregulated fusion protein comprising an output domain and a plurality of input domains, wherein at least one of the input domains is heterologous to the output domain, and the input domains interact with each other to allosterically and external ligand-dependently regulate the output domain.

2. The fusion protein of claim 1, wherein the output domain is catalytic.

6. The fusion protein of claim 1, wherein the plurality comprises four input domains, all heterologous to the output domain, and which form first and second specific binding pairs which allosterically regulate the output domain dependent on first and second, different external ligands, respectively.

8. The fusion protein of claim 6, wherein the input domains cooperatively regulate the output domain as an AND-gate.

14. (Objected To) The fusion protein of claim 1 wherein the output domain is a Neuronal Wiskott-Aldrich Syndrome Protein (N-WASP) WA domain, and the input domains are (i) a PDZ domain and (ii) a SH3 domain.

EVIDENCE APPENDIX

Copies of the following evidence are attached:

Adler et al. (2004) Science's STKE 2004:et1-1

132 Declaration of Dr. Henry Bourne dated Jun 16, 2006.

The above evidence was entered into the record on Jun 17, 2006.

2003: Signaling Breakthroughs of the Year

Elizabeth M. Adler,[1*] Nancy R. Gough,[2] and L. Bryan Ray[3]

(Published 6 January 2004)

Science's STKE rang in 2003 with a new feature on the most notable advances in cell signaling of the past year. The reader response to *2002: Signaling Breakthroughs of the Year* was so overwhelmingly positive that the editors at STKE have decided to make this an annual feature. Thus, we welcome you to 2004 with this article on recent highlights in signaling research. Seven signaling experts were kind enough to share their opinions on what constituted the most exciting research in the area in 2003, paying particular attention to papers likely to blaze the way to new directions in signaling research. This year's participants are Tony Hunter (The Salk Institute, U.S.A.), Ravi Iyengar (Mt. Sinai School of Medicine, U.S.A.), Andre Levchenko (Johns Hopkins University, U.S.A.), Richard Losick (Harvard University, U.S.A.), Mark Ptashne (Memorial Sloan-Kettering Cancer Center, U.S.A.), Eric Vivier (Centre d'Immunologie de Marseille-Luminy, France), and Michael Yaffe (Massachusetts Institute of Technology, U.S.A.). Whereas last year's article emphasized signaling events at the cell membrane, the major themes to emerge this year were structural and organizational, with several experts nominating research concerning the importance of the spatiotemporal organization of cell signaling proteins, and of the role of protein:protein interaction domains in mediating subcellular targeting and determining the response to a given signal.

Eric Vivier noted the importance of "the recent emphasis on the differential utilization of signaling adaptor molecules by a given receptor, or family of receptors" in the context of signaling involving the innate immune response. In particular, Vivier nominated research by Yamamoto *et al.* (*1*) on the Toll-like receptors, which are involved in the recognition of pathogens, and by Diefenbach *et al.* (*2*) on alternately spliced forms of NKG2D, an activating receptor for stress-induced ligands. These papers demonstrate that the identities of the particular adaptors with which a given receptor can associate are crucial to its effector function. In an intriguing variation on this theme, Mark Ptashne nominated two articles from the same group, in which proteins were engineered to mediate novel cellular responses to a particular input. In one article from this group, by Park *et al.* (*3*), a yeast scaffolding protein was engineered to bind a novel combination of kinases, so that the pheromone α-factor, instead of inducing a mating response, initiated a response normally produced by exposure of cells to high osmolarity. In the second article, by Dueber *et al.* (*4*), variants of the actin-regulatory protein N-WASP (neuronal Wiskott-Aldrich signaling protein) were engineered so that they could be activated by a synthetic switch designed by the authors. In nominating this pair of papers, Ptashne noted, "These two remarkable papers show us how, in two quite disparate cases, seemingly intricate and precisely defined protein-protein interactions can be replaced by simpler heterologous interactions without loss of function. These findings shed light on how these systems might have evolved and pave the way for creating new signal-response elements by protein design."

The theme of protein:protein interaction domains, as well as that of the critical importance of spatiotemporal organization to cell signaling, was also raised by Tony Hunter, who nominated the identification of the polo box (*5-7*) and BRCT domains (*8-10*) as novel phosphopeptide binding motifs "as an important advance in understanding signaling by serine/threonine protein kinases." The BRCT domain is a protein-protein interaction motif found in many proteins involved in the response to DNA damage, including BRCA1, a tumor suppressor protein, mutant forms of which are associated with breast and ovarian cancer. BRCT repeats can reportedly interact with sites phosphorylated by DNA damage-activated kinases, such as ATM (ataxia telangiectasia mutated), potentially allowing recruitment of BRCT repeat-containing proteins to sites of DNA damage and repair. The polo box domain, a noncatalytic motif unique to the polo-like kinases, a family of mitotic and checkpoint kinases, allows polo-like kinases to be recruited to appropriate substrates and cellular structures at particular stages of the cell cycle. The implication of both the BRCT and polo box domains in cell cycle- and phosphorylation-dependent protein targeting adds new weight to the emerging role of posttranslational modification in regulating the formation of signaling networks, and provides potential new targets for therapeutic intervention.

Richard Losick nominated research by Shan and Walter (*11*) concerning the mechanisms whereby two bacterial guanosine triphosphatases (GTPases) Ffh and FtsY, which function as a subunit of the bacterial signal recognition particle (SRP) and SRP receptor, respectively—regulate the targeting of secreted proteins to the plasma membrane. These two GTPases, which do not require guanine nucleotide exchange factors, reciprocally activate one another. Shan and Walter showed that the interaction of the two proteins caused a conformational change in FtsY that induced nucleotide-binding specificity. Noting that this was "only the first chapter" in "one of the loveliest stories I have heard in the signal transduction field," Losick offered to keep us apprised as new developments concerning these very unusual GTPases continue to unfold.

Ravi Iyengar nominated two articles indicating that Rap (a GTPase implicated in integrin signaling and known to function as a Ras antagonist) may play a key role in integrating different signaling pathways and in coordinating various physiological responses at the synapse. In the first article, by Morozov *et al.* (*12*), Rap was shown to couple signaling through the adenosine 3′,5′-monophosphate (cAMP) pathway to the regulation of a pool of p42/44 mitogen associated protein kinase (MAPK) and to play a role in mediating both short- and long-term events in-

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volved in the induction of long-term potentiation of synaptic efficacy. In the second article, by Pak and Sheng (*13*), Rap regulation was implicated in the activity-dependent modulation of synaptic structure. Noting that these two studies enhance our "understanding of the role of Rap in the spatiotemporal integration of signals," Iyengar suggested that regulation of Rap activity could thus "integrate synaptic function at multiple levels."

Noting that "although we believe that signal transduction is somewhat stereotypical, with signals proceeding more or less linearly...an ever increasing body of evidence suggests interesting positive and negative feedback possibilities," Andre Levchenko nominated research from two groups, Lorenz *et al.* (*14*) and Corbit *et al.* (*15*), that uncovered an unexpected form of positive feedback. Phosphorylation of Raf kinase inhibitor protein (RKIP) following adrenergic stimulation and activation of protein kinase C causes RKIP to shift from inhibiting Raf-1 to inhibiting G protein-coupled receptor kinase 2 (GRK-2). Both the relief of Raf-1 inhibition and the inhibition of GRK-2 lead to enhanced signaling through the activating receptor and thus represent a novel form of dual positive feedback mediated through phosphorylation of a single protein.

Finally, Michael Yaffe, whose own work on the identification of the BRCT and polo box domains as phosphoserine/threonine binding motifs figured prominently in the foregoing nominations, contributed three nominations concerning research taking place over the last few years. The first was for major advances in understanding TOR (target of rapamycin) signaling. TOR, which plays a key role in regulating cell growth and proliferation, is inhibited by rapamycin, an antifungal agent that is used clinically as an immunosuppressive agent and in cancer therapy. Yaffe cited recent findings identifying TOR-interacting proteins and the link between TOR and phosphoinositide 3-kinase (PI3K) signaling pathways, that "begin to explain how TOR controls cell growth and size, how rapamycin acts, and how insulin might function to control cell growth through the PI3K pathway" (*16-21*). The second nomination concerned the role of phosphorylation-mediated ubiquitin degradation in regulation of the cell cycle, in particular the requirement for multisite phosphorylation of the cyclin dependent kinase inhibitor Sic1 for degradation and thus the transition from G1 to S phase (*22-24*). Finally, Yaffe predicted that the "very recent idea that a persistent DNA damage signal might underlie the mechanism of telomere shortening-initiated senescence" was an important advance that was likely to gain momentum over the next few years (*25-26*).

The STKE editors also put their heads together and had a few suggestions of breaking research. The tumor suppressor p53, which is well known for its nuclear functions in mediating responses to DNA damage, is beginning to be recognized for several nonnuclear activities or functions that extend beyond responding to cellular stress. For example, some of the apoptotic activity of p53 may be occurring through interactions with the mitochondria (*27*). A role for p53 as a Smad partner in signaling by transforming growth factor β also expands the functions of p53 to include regulation of embryogenesis (*28*). As with many proteins, the functions first identified for p53 are only the beginning of multiple diverse activities waiting to be uncovered. Also notable was the accumulation of evidence of a key role for the endoplasmic reticulum in signals causing apoptosis. Although release of factors from the mitochondria has been central to the cell death pathway, a series of articles suggest that the ER and mitochondria actually cooperate to produce a positive feedback system that results in apoptosis (*29-33*)

Related Resources

Editorial Guides

- E. M. Adler, N. R. Gough, L. B. Ray, 2002: Signaling breakthroughs of the year. *Sci. STKE* **2003**, eg1 (2003).
- N. R. Gough, E. M. Adler, Focus Issue: Protein interactions domains, zip codes for delivery. *Sci. STKE* **2003**, eg15 (2003).

Reviews

- T. E. Harris, J. C. Lawrence Jr., TOR signaling. *Sci. STKE* **2003**, re15 (2003).

This Week in ST

- Flexible management. *Sci. STKE* **2003**, tw75 (2003).
- Modular switches. *Sci. STKE* **2003**, tw377 (2003).
- Risk factors in breast and ovarian cancer. *Sci. STKE* **2003**, tw421 (2003).
- Looking for like-minded partners. *Sci. STKE* **2003**, tw86 (2003).
- From electrical activity to dendritic spine morphology. *Sci. STKE* **2003**, tw458 (2003).
- Killing two kinases with one inhibitor. *Sci. STKE* **2003**, tw473 (2003).
- Raptor forms a nutrient-sensitive complex with mTOR. *Sci. STKE* **2002**, tw277 (2002).
- Controlling signal input to mTOR. *Sci. STKE* **2003**, tw317 (2003).
- Mitochondrial p53. *Sci. STKE* **2003**, tw134 (2003).
- TGF-β and p53 partner up. *Sci. STKE* **2003**, tw190 (2003).
- Unmixing apoptotic signals. *Sci. STKE* **2003**, tw141 (2003).
- Prions stress out the ER. *Sci. STKE* **2003**, tw409 (2003).
- Cytochrome c and IP_3R: A deadly handshake. *Sci. STKE* **2003**, tw471 (2003).
- Cleaved GSPT1: A new IAP-binding protein. *Sci. STKE* **2003**, tw396 (2003).
- Getting SET for cell death. *Sci. STKE* **2003**, tw112 (2003).

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Title: *Protein Logic Gates*

DECLARATION UNDER 37CFR1.132

I, Professor Henry Bourne declare and state as follows:

1. I am a Professor in the Department of Cellular and Molecular Pharmacology at the University of California, San Francisco. I received my MD degree from Johns Hopkins and took my postdoctoral training at the National Institutes of Health and UCSF. I became a faculty member at UCSF in the Department of Medicine in 1971, and served as chair of the Department of Pharmacology from 1984 to 1992. I have authored numerous scientific papers in the field of protein engineering. I am familiar with this patent application and the related ongoing work in the Lim laboratory.

2. The claimed invention is an autoregulated fusion protein comprising an output domain and a plurality of input domains, wherein at least one of the input domains is heterologous to the output domain, and the input domains interact with each other to allosterically and external ligand-dependently regulate the output domain. The fusion proteins link protein input and output domains that are normally not related to provide protein signaling switches analogous to logic gates with diverse and novel input/output properties.

The selected input and output domains are discretionary to the user according to intended use, and essentially any output domain providing a desired activity or binding affinity may be employed, so long as output activity can be regulated by ligand-dependent interaction of the input domains (e.g. Specification, p. 6, lines 13-15). Similarly the selection of input domains is user discretionary, so long as the selected domains interact to provide the requisite ligand-dependent gating of the output domain (e.g. Specification, p.7, lines 18-19).

For example, output domain functional compatibility with the fusion proteins is readily confirmed in routine activity screens (e.g. Specification, p.6, lines 16-17). A wide variety of output activities may be obtained, depending on the ultimate user application, including catalytic, label-generative, metabolic, apoptotic, and specific-binding output activities (Specification, p.6, lines 17-19). Table 1 lists exemplary output domains shown to have regulatable output activities, including well-studied kinase, phosphatase and protease domains (Specification, p.6, line 25 – p.7, line 7).

Similarly, input domain functional compatibility (demonstrating gating behavior) with the fusion proteins is readily confirmed in routine activity screens. A wide variety of interacting input domains may be used, depending on the ultimate user application, including peptide hormones and cognate receptor ligand binding domains (LBD), immune receptors and cognate antigenic peptides, src-homology domains and cognate peptide ligands, and various catalytic input domains, including modular proteases and both cleavable and non-cleavable pseudosubstrate peptides, modular kinases and peptide substrates, modular phosphatases and phospho-peptide substrates, etc. The input domain interaction can be provided by homo- or hetero-dimerization, by specific pair binding, by higher order complex formation, by enzyme-substrate catalysis (e.g. phosphorylation, glycosylation, prenylation, acylation, lipid modification, etc.). Specification, p.7, lines 18-30.

Preferred input domains comprise native, modular interacting domains which mediate binding of naturally interacting proteins, or natural, modular receptors or enzymes and their cognate ligands and substrates. A wide variety of such modular interacting components has been identified, categorized and subject to grafting. In addition, suitable input domains may be derived from vast public databases of known interacting proteins, including Database of Interacting Proteins (DIP), Database of Ligand-Receptor Proteins, Java-based DIP, and LiveDIP; see, e.g. Xenarios, et al. (2002) NAR 30:303-5; Xenarios, et al.(2001) NAR 29:239-41; Xenarios et al., (2000) NAR 28:289-91; Deane et al.(2002) Mol Cell Prot 1:349-356; Graeber et al. (2001) Nat.Genet. 29:295-300; Marcotte et al. (2001) Bioinformatics 17:359-63; Salwinski et al. (2003) Mol Cell Proteomics. 2002 May;1(5):349-56; Xenarios et al. (2001) Curr Opin Biotechnol 12:334-339. In addition, many protein interaction domains can be mutated to provide alternative specificity binding partners. For example, mutation of a threonine residue of the Src SH2 domain to tryptophan converts ligand-binding specificity from the Src-like pTyr-Glu-Glu-Ile (SEQ ID NO:1), to the signature Grb2 binding motif pTyr-X-Asn (Kimber et al. Molecular Cell 2000. 5, 1043-1049). Table 2 lists exemplary input domain binding pairs shown to have external ligand regulatable binding. Specification, p. 8, line 5 – p.18, line 22).

To promote their interactions, one or more of the input domains may be coupled to the fusion protein through a linker or spacer peptide. Linker peptides are widely used in fusion proteins. Linker sequence and length are user-discretionary, though the linkers should not interfere with the output domain when the switch is in the active state (e.g. de-repressed), which is readily confirmed empirically. Preferred linkers often provide structural flexibility and mobility to the input domain. Exemplary use of linker peptides is provided in the disclosed examples of exemplary fusion proteins. Specification, p.7, lines 31 – p.8, line 4.

The claimed protein switches are readily designed or screened such that external ligand activation up-regulates, down-regulates, or otherwise alters output activity. For example, activation can increase, decrease or alter label expression, binding or substrate affinity or specificity, etc. In particular embodiments, the output domain is constitutively active or functional, and in the absence of the ligand, the input domains interact to inhibit the output domain. Where the selected output domain also comprises a suitable input or interaction domain, this endogenous interaction domain may be exploited to create novel allostery in conjunction with a heterologous input or interaction domain. Typically, such endogenous input domains are positioned on the output domain so as to not interfere with the output activity, e.g. the output activity when the fusion protein is de-repressed with ligand. Specification, p.7, lines 8-17.

A wide variety of external ligands may be used to activate the switches by interacting with one or more of the input domains. The external ligands may activate reversibly, such as by reversible competitive or allosteric interaction with one or more of the input domains, or may

activate irreversibly, such as through covalent modification. For example, in the case of an SH3 input domain, proline rich peptides can be used as both a second, integral input domain, and as an external competitive ligand. Alternatively, the external ligand can comprise a kinase activity which phosphorylates (covalently modifying) the SH3 domain proximate to the proline-rich binding site, and thereby disrupts interaction of the input domains. Specification, p.18, line 24 – p.19, line 2.

In particular embodiments, the fusion proteins comprise two input domains, both heterologous to the output domain, and which form a specific binding pair. In these embodiments, the input domains may also be referred to as receptor-ligand pairs, wherein this internal ligand is one of the input domains, as opposed to the actuating, external ligand which competitively or allosterically disrupts pair-specific binding of the input domains. This input domain binding pair motif may be expanded with additional input domains to provide any desired form of cooperative or antagonistic regulation. For example, the fusion protein may comprise two or more specific binding pairs of input domains which provide higher-order cooperative gating behavior. Accordingly, depending on design or selection, multiple input domains can cooperatively regulate the fusion protein in a wide variety of functionalities, including as an OR-gate, an AND-gate, and an AND-NOT-gate. Similarly, a plurality of output domains can be combined in a single fusion protein, to provide more complex switching. Table 3 provides the compositions of exemplary fusion protein switches, including their corresponding output domain, input domains and regulating external ligand. Specification, p.19, lines 3-24.

Those skilled in the art have recognized that the invention is not limited to a single embodiment, but that Applicants' teachings "...pave the way for creating new signal-response elements by protein design. Adler et al. Signaling Breakthroughs of the Year. Adler, Gough, and Ray (2004) Science's STKE 2004: eg1-1. In fact, following the guidance of their disclosure, the inventors have used modular intramolecular interaction domains to engineer novel regulatory control over distinct catalytic output activities. For example, they have demonstrated the ability to apply their method of regulation to Dbl-homology (DH) domains of distinct guanine nucleotide exchange factors (GEFs). They have inserted the engineered GEF switches into cells and used them to precisely alter cell behavior, conferring a new morphological response when stimulated by an appropriate upstream target. They have also demonstrated that they can build these types of autoinhibitory switches that respond to diverse input stimuli. For example, they have engineered switch proteins that are activated by a specific protein kinase by using an autoinhibitory control unit that consists of a PDZ domain-peptide recognition pair whose interaction is disrupted by phosphorylation. They have also built switches with more complex behavior by combining multiple domains. For example, they have built switches that are controlled by 3 inputs simultaneously (by using 3 autoregulatory domain interactions) or switches that show digital type (all-or-none) responses by using multiple autoregulatory interactions of the same type. I am familiar with these types of protein engineering, and in my opinion all these results were predictably obtained following the guidance of their patent application without requiring any undue experimentation.

Accordingly, in my opinion the Specification enables one skilled in the art to make and use without undue experimentation an autoregulated fusion protein comprising an output domain and a plurality of input domains, wherein at least one of the input domains is heterologous to the output domain, and the input domains interact with each other to allosterically and external ligand-dependently regulate the output domain. The Specification enables one skilled in the art to make and use such fusion proteins with a wide range of alternative output and input domains. Swapping alternative input and output domains in the recited fusion proteins involves only routine gene splicing and activity screening.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful, false statements may jeopardize the validity of the application and any patent issuing therefrom.

Date: 16 June '06

Henry R Bourne

Henry Bourne, M.D.

RELATED PROCEEDINGS APPENDIX

No related proceedings are known to exist.